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Biosynthesis of Murine Terminal Deoxynucleotidyltransferase*

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An immunoprecipitation assay for measuring synthesis of murine terminal deoxynucleotidyltransferase (EC 2.7.7.31) has been developed using rabbit antiserum to calf terminal transferase. The antiserum precipitates a single M_r = 60,000 polypeptide (TdT-60) from all cell lines and tissues that contain enzymologically demonstrable terminal transferase. This polypeptide is not precipitated from labeled extracts of cells that lack terminal transferase by enzymological criteria. TdT-60 fractionates with terminal transferase during phosphocellulose chromatography and sediments with it in a sucrose gradient. TdT-60 is not detectably processed to lower molecular weight polypeptides, and terminal transferase activity sediments as a M_r = 60,000 activity; thus, we believe it to be the active form of terminal transferase. Using this assay we have demonstrated that terminal transferase is synthesized in both the murine thymus and the bone marrow at a rate proportional to its biochemically measured steady state level. After cortisone treatment of mice, the M_r = 60,000 polypeptide disappears from the thymus and then reappears as the thymus begins to be repopulated.

Terminal deoxynucleotidyltransferase (nucleoside triphosphate: DNA nucleotidylexotransferase; EC 2.7.7.31) is an unusual DNA polymerase that can catalyze the addition of deoxyribonucleotides onto the 3'-OH end of an oligodeoxynucleotide primer in the absence of a directing template (1-3). It is normally found only in immature thymic lymphocytes (4-9) and in bone marrow lymphocytes (6, 7, 10). Bone marrow terminal transferase[1] is restricted to larger, less dense cells (11, 12), at least some of which are committed to the T-lymphocyte pathway and may be prothymocytes (13, 14). The enzyme is also found in circulating lymphocytes during particular leukemic disease states (6, 7, 9, 15-22) and in the spleens of athymic nu/nu mice (23, 24). All of these observations have been made using standard biochemical assays and many of them have been confirmed by both radioimmunoassay (25) and immunofluorescent assays (23, 26-28).

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[1] The abbreviations used are: terminal transferase, terminal deoxynucleotidyltransferase; TdT-60, M_r = 60,000 protein immunoprecipitated with antiserum prepared against terminal transferase; PBS, 1% Triton X-100, 10 mM sodium PO_4 (pH 7.5), and 100 mM NaCl.

The detection of terminal transferase in lymphoid cells of a particular tissue does not, however, constitute proof that cells of that tissue are synthesizing terminal transferase. For example, it has been suggested that terminal transferase found in normal bone marrow is due to migration of terminal transferase-containing cells from the thymus to the marrow (14). A similar migration explanation has been advanced to explain the presence of terminal transferase in some abnormally inflamed tonsils (29) and in the spleens of athymic nu/nu mice (13, 24). An assay for the actual metabolic synthesis of terminal transferase would be valuable for answering such questions. Such an assay could also determine whether terminal transferase is synthesized as two distinct polypeptide chains, which then associate into the dimer form found for purified calf terminal transferase (25, 30, 31), or whether there is processing of a larger entity to yield functional terminal transferase, as suggested by Johnson and Morgan (32).

We report here the development of an immunoprecipitation assay for the metabolic synthesis of murine terminal transferase using a rabbit anti-calf terminal transferase serum. With this assay we have demonstrated that murine terminal transferase is synthesized as a single polypeptide of M_r = 60,000 that is not processed to another detectable form. We also show that terminal transferase is synthesized in both murine thymus and bone marrow at rates proportional to its biochemically measured steady state level. While this manuscript was in preparation, Bollum and Brown (33) reported that immunoprecipitation of human lymphoblastoid tumor cells with a rabbit anti-calf terminal transferase precipitated a major protein band of M_r = 58,000.

MATERIALS AND METHODS

Preparation of Terminal Transferase—Three milligrams of terminal transferase were purified from 30 kg of calf thymus in collaboration with the Tufts New England Enzyme Center (Boston, MA). The initial steps in the purification duplicated the methods of Yoneda and Bollum (34) and Chang and Bollum (30). Tissue was homogenized in NaCl/KPO_4 buffer, pH 7.2, and clarified by low speed centrifugation. The supernatant was acidified to pH 6.5 and then adsorbed to phosphocellulose (Whatman). The enzyme was eluted from the resin by increasing the pH to 7.2 and the phosphate concentration to 250 mM. The pooled peak then was passed through a Whatman DE11 (DEAE-cellulose) column to remove nucleic acids. The resulting material was dialyzed and concentrated using an ultrafiltration apparatus and then was adsorbed to a second phosphocellulose column. Enzyme was again batch-eluted with high phosphate and the pooled peak was collected as a 35 to 55% ammonium sulfate fraction. The enzyme then was resuspended and treated with cacodylic acid, pH 4.5, to inactivate and remove other DNA polymerases. The supernatant, containing terminal transferase activity, was again concentrated with ammonium sulfate. This material was suspended in acid phosphate, dialyzed into 50 mM Tris·HCl (pH 7.7), 1 mM EDTA, 1 mM 2-mercaptoethanol, and adsorbed and eluted from phosphocellulose using a linear KCl gradient as previously described (15). The resulting peak fractions were pooled and fractionated on a Sephadex G-100 (Pharmacia) column, using 12 mM phosphate buffer, pH 7.2, and 1 mM dithiothreitol. The peak material, with a mean molecular weight

of approximately 35,000, then was adsorbed to an oligo(dT)-cellulose resin (T-2, Collaborative Research, Waltham, MA), in a manner similar to that described by Okamura *et al.* (31), except that the adsorption was done with 10 mM KH_2PO_4/K_2HPO_4 (pH 7.2), 1 mM dithiothreitol, 50 mM KCl, and 20% v/v glycerol. Terminal transferase activity was eluted from this column using a linear salt gradient. This material was used as a source of antigen in most cases. In some cases, a more highly purified enzyme preparation was made from the oligo(dT)-purified material using hydroxylapatite chromatography (30). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the various fractions during purification showed that by Coomassie blue staining bands of M_r = 28,000 and 8,000 became major components only after the oligo(dT)-cellulose chromatography (Fig. 1). These two bands have previously been shown to constitute purified calf terminal transferase (30). The material purified through hydroxylapatite was even purer than the oligo(dT)-purified fractions although the limited amount available did not allow as much to be analyzed (Fig. 1, *Lane D*).

Preparation of Antiserum—Antiserum to calf terminal transferase was prepared by injecting two rabbits with alum-adsorbed terminal transferase as described previously for immunization of mice (25). Initial immunizations were done with oligo(dT)-purified enzyme (170 μg/rabbit) and the first booster immunizations were done with hydroxylapatite-purified material (75 to 100 μg of terminal transferase protein/rabbit). Successive booster immunizations were done with 100 μg of oligo(dT)-purified terminal transferase every 3 to 4 weeks. The antisera used in our studies inhibited calf terminal transferase enzyme activity more than 70% at a dilution of 1:80 in an assay identical to that described in Kung *et al.* (25). Like the mouse sera used previously (25), these rabbit sera did not inhibit normal murine DNA polymerases-α and -β.

Cell Lines—RLδ11 is a cloned subline derived from *in vitro*-passaged RLδ1, a radiation-induced BALB/c leukemia, derived by Dr. E. A. Boyse (28). 18-48 and 18-81 are clones derived from leukemic cell lines induced *in vitro* from BALB/c bone marrow cells with Abelson murine leukemia virus by Dr. N. Rosenberg (28, 35). CEL-42 is a clone derived from an *in vitro*-passaged EL4 tumor donated to the MIT Cell Culture Center. Cells were isolated from spleen, thymus, and bone marrow as previously described (13) except that a buffer developed by Dr. V. Sato (Harvard University) was used to prevent cell clumping (Eagle's minimal essential medium with Hank's salts and L-glutamine, without sodium bicarbonate (Gibco 410-1200), supplemented with 1 mM $MgCl_2$ and 1 mM sodium phosphate and adjusted to pH 7.05).

In cases where there were large numbers of dead cells (as in the cortisone experiments), viability was increased by suspending the cells in a 35% bovine serum albumin solution (Miles, Pathocyte 5), and overlaying with a 10 or 15% bovine serum albumin solution in a nitrocellulose centrifuge tube (Beckman). This step gradient was then centrifuged to equilibrium at 15,000 to 16,000 rpm for 30 min in a swinging bucket rotor (SW 41 or SW 50.1) at 4°C. Viable cells band at the density interface, whereas dead cells sediment to the bottom of the tube. Recovery is more than 80% and resulting viability greater than 95%.

Biosynthetic Labeling and Immunoprecipitation—In most cases 10^7 cells were washed with Dulbecco's modified Eagle's medium lacking serum and methionine and then suspended in the same medium in a 2-ml volume with 100 to 200 μCi of [^{35}S]methionine (New England Nuclear Corp.). Following the labeling period, the cells were harvested by low speed centrifugation and the supernatant was removed. Lysates were prepared for immunoprecipitation using the phosphate and detergent lysis buffer described in Witte and Baltimore (36). This buffer contains 10 mM $NaPO_4$ (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate. In many cases, 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 mM EDTA were added to the lysis buffer to minimize proteolysis.

The lysates were reacted with 5 μl of deaggregated antiserum for 16 to 20 h at 4°C and antigen·antibody complexes were precipitated

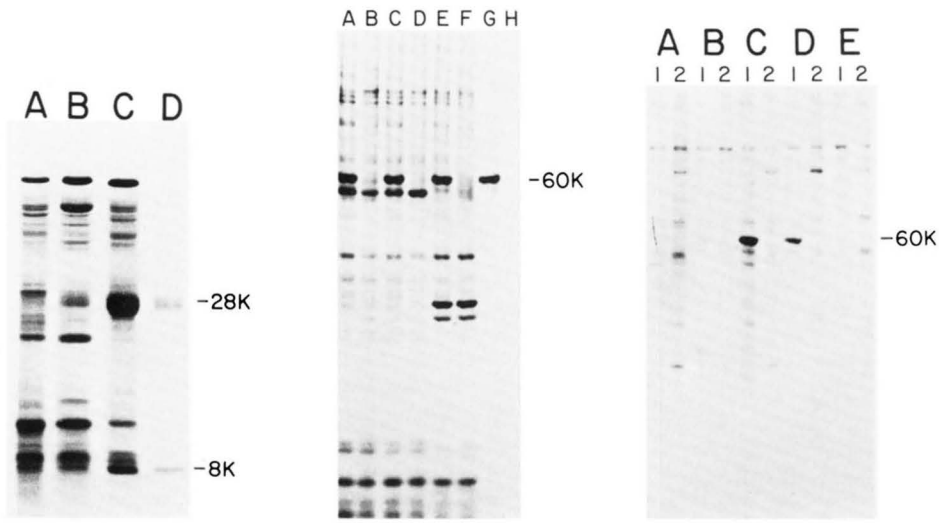

FIG. 1 (*left*). **The purification of calf terminal transferase.** Samples at various stages in the purification of calf enzyme containing 5 to 50 μg of total protein were dissociated by boiling in gel sample buffer, then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis using the method of Laemmli (38) on a 5 to 20% linear slab gel. The gel was stained with Coomassie brilliant blue, destained, and fixed with 10% acetic acid, 10% methanol. *A*, pooled peak material from third phosphocellulose column (salt gradient elution); *B*, pooled peak material from G-100 column; *C*, pooled peak material from oligo(dT)-cellulose column; *D*, pooled peak material from hydroxylapatite chromatography.

FIG. 2 (*center*). **Immunoprecipitation and electrophoretic analysis of terminal transferase in a murine tumor cell line.** RLδ11 tumor cells were metabolically labeled for 1 h with 50 μCi/ml of [^{35}S]methionine, and cell extracts were immunoprecipitated with rabbit anti-calf terminal transferase serum or normal rabbit serum. Each channel represents labeled protein from 5 × 10^5 cells. Samples were denatured in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and analyzed on a sodium dodecyl sulfate/10% acrylamide gel (38) and developed by fluorography (39). *A*, sample treated and precipitated with anti-terminal transferase in 1% Triton X-100, 10 mM $NaPO_4$ (pH 7.5), and 100 mM NaCl (PBS); *B*, sample treated and precipitated with normal rabbit serum in 1% Triton X-100, 10 mM $NaPO_4$ (pH 7.5) + 100 mM NaCl; *C*, sample treated and precipitated with anti-terminal transferase in PBS/0.1% sodium dodecyl sulfate; *D*, sample treated and precipitated with normal rabbit serum in PBS + 0.1% sodium dodecyl sulfate; *E*, sample treated and precipitated with anti-terminal transferase in PBS + 0.1% sodium dodecyl sulfate + 0.5% deoxycholate; *F*, sample treated and precipitated with normal rabbit serum in PBS + 0.1% sodium dodecyl sulfate in 0.5% deoxycholate; *G*, sample treated and precipitated with anti-terminal transferase in PBS + 0.1% sodium dodecyl sulfate + 0.5% deoxycholate + 1% Triton X-100; *H*, sample treated and precipitated with normal rabbit serum in PBS + 0.1% sodium dodecyl sulfate + 0.5% deoxycholate + 1% Triton X-100.

FIG. 3 (*right*). **Immunoprecipitation and electrophoretic analysis of terminal transferase in various murine tumor lines.** Tumor cells metabolically labeled with [^{35}S]methionine as described under "Materials and Methods" and cells were extracted and immunoprecipitated with 5 μl of anti-terminal transferase (*Lane 1*) or normal rabbit serum (*Lane 2*). Samples were collected and analyzed as in Fig. 2. *A*, 18-81; *B*, 18-48; *C*, RLδ11; *D*, CEL-42; *E*, HRS-ST34.

using whole formalin-fixed *Staphylococcus aureus* according to the method of Kessler (37). Precipitates were washed with lysis buffer, resuspended in sample buffer (50 mM Tris-HCl (pH 6.8), 2.5% 2-mercaptoethanol, 1% sodium dodecyl sulfate), and boiled for 2 min. Reduced samples were analyzed on sodium dodecyl sulfate-polyacrylamide gels of varying concentrations according to the method of Laemmli (38). Gels were stained with Coomassie brilliant blue R250 (Eastman), fixed in 10% acetic acid, 10% methanol, and fluorographed according to Bonner and Laskey (39).

Phosphocellulose Chromatography of Terminal Transferase and Enzyme Assays—Terminal transferase and DNA polymerase-α (EC 2.7.7.7) were partially purified from crude cell extracts, as previously described (13), by adsorbing the enzymes to phosphocellulose resin and then eluting them with a linear KCl gradient. Enzyme assays used for terminal transferase and DNA polymerase-α were as previously described (13, 28). DNA polymerase-α was measured using a mixture of poly(dC) and poly(dI) as a template-primer combination (Miles) and measuring the incorporation of [^{3}H]dGMP (New England Nuclear Corp.) into acid-precipitable material in the presence of Mg^{2+} ion (15). Under these conditions, murine terminal transferase will not catalyze the incorporation of label. Terminal transferase was assayed by the incorporation into acid-precipitable material of [^{3}H]dGMP or [^{32}P]dGMP in the presence of an oligo(dA)$_{12-18}$ (Collaborative Research) primer and Mn^{2+} ion.

Cortisone Treatment—Mice were injected with 2.5 mg of cortisone acetate (Upjohn Co.) as previously described (6). Cell suspensions were made from mice killed at the appropriate time after injection.

RESULTS

Anti-terminal Transferase Precipitates a M_r = 60,000 Protein—To determine whether rabbit antiserum to calf terminal transferase could be used to analyze the metabolism of terminal transferase in mice, we examined the ability of the serum to immunoprecipitate proteins from extracts of metabolically labeled cells. *In vitro*-propagated RL♂11 mouse tumor cells, known to contain a high terminal transferase level by biochemical assay[2] (28), were labeled with [^{35}S]methionine. Cell extracts were exposed to antiserum, the resulting antigen·antibody complexes were collected by binding to *Staphylococcus aureus*, and the precipitates were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described elsewhere (36). A reproducible, major band of protein of M_r = 60,000 (TdT-60) was observed when extracts were precipitated with anti-terminal transferase in the presence of three detergents (Fig. 2, *Lane G*). Normal rabbit serum did not immunoprecipitate any detectable TdT-60 under these conditions (Fig. 2, *Lane H*). The three detergents in the immunoprecipitation buffer were needed to maintain nonspecific precipitation at an acceptably low level; various other combinations of the detergents gave more bands that were precipitated by both immune and normal serum (Fig. 2, *Lanes A* to *F*).

Because purified calf terminal transferase is known to have two subunits of M_r = 27,000 and 8,000 (25, 30), the finding of a single polypeptide of M_r = 60,000[3] was surprising and we, therefore, designed a number of experiments to examine whether the band was truly terminal transferase.

To ask whether the presence of enzymatically detected terminal transferase correlated with the presence of the TdT-60 band, we studied a series of murine lymphoid tumor lines containing varying levels of terminal transferase. Lines RL♂11 and CEL-42 contained easily detectable terminal transferase; the Abelson virus-induced line, 18-48, contained a low but measurable quantity of terminal transferase, and both the Abelson virus-induced line, 18-81, and the line derived from a spontaneous tumor, HRS-ST34, lacked detectable terminal transferase[2] (28). Occurrence of TdT-60 correlated with the enzymatically determined levels of terminal transferase (Fig. 3): immune serum (*Lane 1*) but not normal rabbit serum (*Lane 2*) precipitated dark M_r = 60,000 bands from RL♂11 and CEL-42 (Fig. 3, *C* and *D*), a light band from 18-48 (Fig. 3*B*), and no M_r = 60,000 band from HRS-ST34 or 18-81 (Fig. 3, *E* and *A*). With continuous passage, CEL-42 lost enzymatically detected terminal transferase as well as TdT-60; by 150 generations both assays showed no detectable terminal transferase (data not shown). By contrast, RL♂11 cells maintained terminal transferase by both assays for more than 400 cell divisions.

Relationship of TdT-60 and Terminal Transferase Enzyme—To examine whether terminal transferase enzymatic activity and TdT-60 would co-purify, an extract of [^{35}S]methionine-labeled RL♂11 cells was chromatographed through phosphocellulose using a linear KCl gradient, and the fractions were assayed for enzyme activity. Although the ^{35}S-labeled proteins eluted broadly, terminal transferase eluted as a single peak (Fig. 4*A*) at a KCl concentration of 0.27 M KCl, which is in agreement with previous results (6, 23). Immunoprecipitation of the peak fraction of enzyme activity showed a strong M_r = 60,000 band (Fig. 4*C*); fractions outside the enzyme peak had no specifically immunoprecipitable band (data not shown).

The antiserum used for precipitation was made against terminal transferase purified by use of phosphocellulose chromatography and, thus, the finding of TdT-60 in the terminal transferase peak eluted from phosphocellulose does not prove whether TdT-60 is terminal transferase or a contaminant. To examine this question, we turned to a fractionation step not used in the terminal transferase purification, sucrose gradient sedimentation. The peak terminal transferase fractions eluted when the ^{35}S-labeled preparation was passed through phosphocellulose were pooled and sedimented through a sucrose gradient. The enzyme activity sedimented just behind a M_r = 68,000 bovine serum albumin marker but well ahead of a M_r = 43,000 ovalbumin marker (Fig. 4*B*). Thus, the enzyme behaved like a M_r = 60,000 protein (and not like the M_r = 36,000 purified calf thymus terminal transferase). A variety of fractions were selected from the sucrose gradient and analyzed by immunoprecipitation for their content of TdT-60 (Fig. 4*C*). Only the peak enzyme fractions had TdT-60. Thus, we can conclude that TdT-60 co-sediments with terminal transferase and that the size of terminal transferase by sedimentation is consistent with a M_r = 60,000.

Another test of the relationship of TdT-60 to terminal transferase enzyme was to compete its immunoprecipitation with purified calf terminal transferase. Enzyme purified by oligo(dT)-cellulose chromatography was mixed with extracts of terminal transferase-positive mouse cells and completely prevented precipitation of the M_r = 60,000 band (data not shown).

As a final test, the ability of the immunoprecipitation procedure to specifically remove terminal transferase from crude extracts of terminal transferase-positive mouse cells was examined. The extracts were exposed to anti-terminal transferase serum or normal rabbit serum, the antigen·antibody complexes were removed by binding to *S. aureus*, and the remaining proteins were chromatographed on phosphocellulose. Enzyme assays showed that without added serum (Fig. 5*A*) or with normal rabbit serum (Fig. 5*B*) equal amounts of terminal transferase and normal cellular DNA polymerase-α were present. The specific antiserum, however, removed terminal trans-

[2] A. Silverstone, L. Sun, O. N. Witte, and D. Baltimore, unpublished data.

[3] The actual molecular weight of the TdT-60 polypeptide appears to be 55,000 to 60,000, depending on the electrophoresis system used and the markers chosen. Because determination of molecular weight by electrophoresis is not always accurate, we have designated the protein TdT-60 for convenience but its true molecular weight may be somewhat different and possibly nearer 55,000.

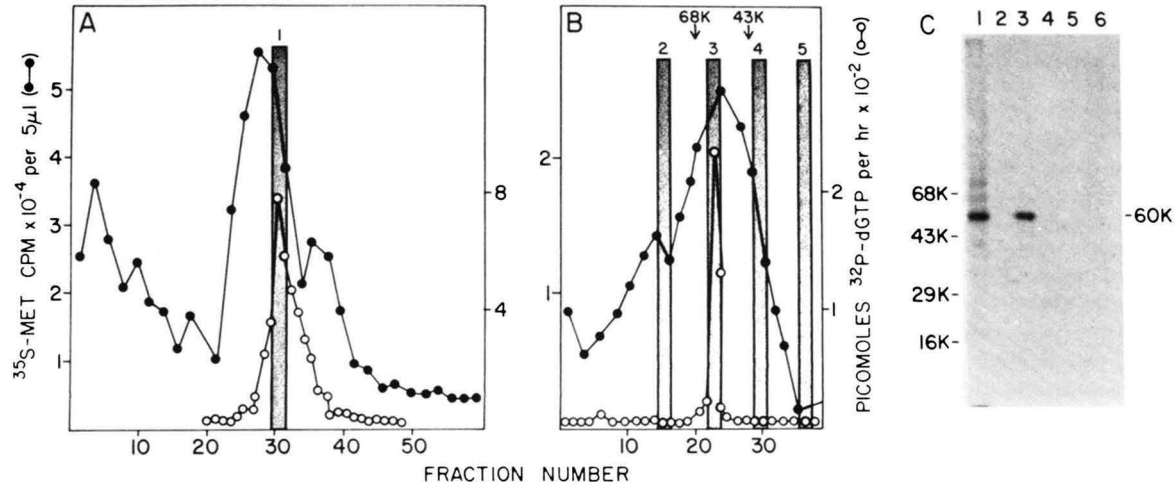

FIG. 4. **Co-purification of terminal transferase activity and TdT-60 by ion exchange chromatography and glycerol gradient centrifugation.** *A*, thymocytes (3×10^9) from C57BL/6 mice were labeled with 2 mCi of [^{35}S]methionine (New England Nuclear, Boston, MA) for 1 h as described under "Materials and Methods." Cells were washed, pelleted, frozen, extracted, and prepared for chromatography as previously described (13). The extract was adsorbed to phosphocellulose in 50 mM KCl and then eluted with a gradient of 100 mM to 1 M KCl. Fractions (0.4 ml) were analyzed for total [^{35}S]methionine counts/5 μl (●——●) and for terminal transferase activity as picomoles/h of each fraction employing [^{32}P]dGTP (1000 Ci/mmol; Amersham, Chicago, IL) in reaction conditions previously described (13). *B*, material from the terminal transferase peak fractions (*hatched area 1*) was dialyzed into 0.1 M K_2HPO_4/KH_2PO_4 (pH 7.5), 1 mM 2-mercaptoethanol, and 5% sucrose and analyzed on a linear 5 to 20% sucrose gradient (SW 50.1 tube, Beckman Instruments, 4°C, 42,000 rpm, 20 h) (4). Markers of M_r = 68,000 (bovine serum albumin) and 43,000 (ovalbumin) were run on a parallel gradient. Fractions (0.13 ml) were analyzed for total [^{35}S]methionine counts/5 μl (○——○) and for terminal transferase activity as picomoles of [^{32}P]dGTP incorporated/h/fraction using a cacodylate buffered assay (2, 13). *C*, equal volumes of material from each of the hatched areas (*1* to *5*) were extracted, immunoprecipitated with 5 μl of rabbit anti-terminal transferase serum, and analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and fluorography as described under "Materials and Methods." *Lane 6* represents a pool of *Samples 1* to *5* immunoprecipitated with normal rabbit serum. The fluorogram was exposed for 5 days.

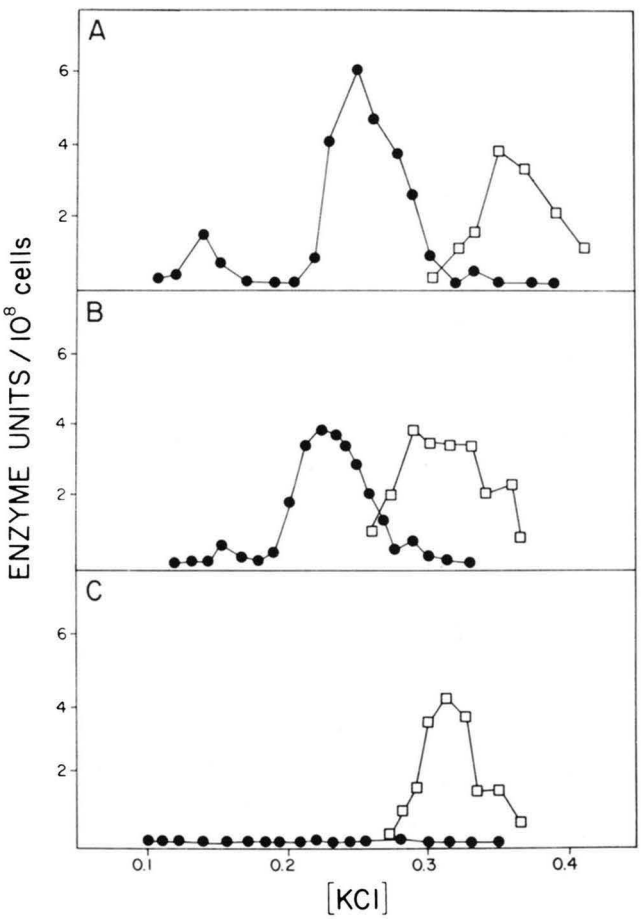

FIG. 5. **Precipitation of murine terminal transferase activity by anti-calf terminal transferase antiserum and *S. aureus*.** Early passage frozen CEL-42 cells, 4×10^8, were thawed and extracted with phenylmethylsulfonyl fluoride, Triton X-100, and KCl and dialyzed into low salt. A 100,000 × *g* supernatant was prepared and divided into 3-ml aliquots for treatment as below. *A*, no addition. These were allowed to stand overnight. *S. aureus* was added in PBS to each and, after 60 min, antigen-antibody complexes were spun down. The supernatants were clarified and then adsorbed with phosphocellulose. Enzyme was eluted with a KCl salt gradient, and activity of DNA polymerase-α (□) and terminal transferase (●) was measured (as referenced under "Materials and Methods"). *B*, 330 μl of normal rabbit serum was added. *C*, 330 μl of anti-terminal transferase serum.

ferase from the extract while not reducing DNA polymerase-α below the level left by normal rabbit serum (Fig. 5*C*).

Therefore, by the correlation of the presence of terminal transferase enzyme and TdT-60 in a series of tumor cell lines, by sedimentation of terminal transferase activity and TdT-60, by the ability of terminal transferase to block precipitation of TdT-60, and by the ability of the anti-terminal transferase serum to precipitate terminal transferase activity selectively, we conclude that TdT-60 is mouse terminal transferase. Although we have not had enough starting material to purify mouse terminal transferase to homogeneity, Ihle and Pazmino (Frederick Cancer Research Center, Maryland) found that terminal transferase partially purified from thymus tissue of 2,000 mice had a molecular weight between 60,000 and 70,000 by gel exclusion chromatography.[4]

Stability of the Metabolically Labeled Product—Because purified calf terminal transferase is composed of two chains (30), we examined if TdT-60 would be processed in cells. Early passage CEL-42 cells were labeled with [^{35}C]methionine for 15 min and then resuspended in unlabeled medium. The pulse-labeled cells and cells chased for 1 or 3 h had approximately equal TdT-60 bands and there was no appearance of specifically precipitable bands other than TdT-60 (Fig. 6*A*). With pulse-labeled RL♂11 cells, chases of 4, 10, or 24 h showed extensive loss of radioactivity from TdT-60 but no new labeled protein or cleavage fragments were observed.

[4] J. N. Ihle and N. H. Pazmino, personal communication.

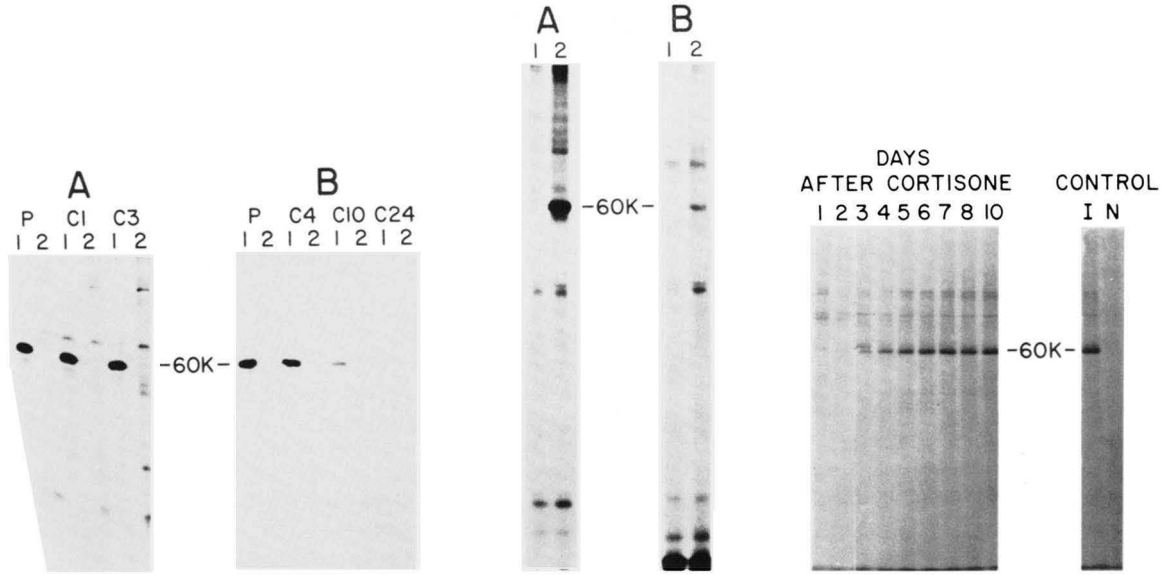

FIG. 6 (*left*). **Pulse chase labeling of RLδ11 and CEL-42 cells.** CEL-42 or RLδ11 cells were incubated at 37°C for 15 min in met-Dulbecco's modified Eagle's medium + 50 μCi/10^7 cells of [^{35}S]methionine, then pelleted and resuspended in complete medium with heat-inactivated fetal calf serum (20%). Samples were taken at the indicated times (h) and extracted for immune precipitation with anti-terminal transferase (*Lane 1*) or normal rabbit serum (*Lane 2*). *A*, CEL-42 cells; *B*, RLδ11 cells.

FIG. 7 (*center*). **Tissue distribution of TdT-60.** Cell suspensions were prepared from (*A*) thymus and (*B*) bone marrow. Lymphoid cells, 2×10^7, from each tissue were incubated 1 h with 150 μCi of [^{35}S]methionine in medium lacking methionine as previously described. Cells were lysed and processed for analysis in the presence of freshly added 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out from 2×10^6 acid-precipitable counts of material from each tissue. *Lane 1*, precipitation with normal rabbit serum; *Lane 2*, precipitation with anti-terminal transferase.

FIG. 8 (*right*). **Effect of cortisone upon terminal transferase synthesis in the thymus.** Mice were inoculated with 2.5 mg of cortisone acetate and killed at various times after injection. Thymus cell suspensions were prepared and cells were metabolically labeled. Extracts and preparation of immunoprecipitates were all carried out in the presence of 1 mM phenylmethylsulfonyl fluoride. Dead cells were removed before labeling with a discontinuous 15:35% bovine serum albumin gradient as described under "Materials and Methods." All precipitations were carried out from 10^6 acid precipitable counts of labeled material. *Days 1* to *10*, with anti-terminal transferase. *Control*: *I*, with anti-terminal transferase; *N*, with normal rabbit serum. Exposure was for 7 days.

Tissue Distribution of TdT-60 Synthesis—Terminal transferase enzyme activity has been detected in thymus and bone marrow of normal mice (4, 6, 11) but not in spleen or other peripheral lymphoid tissue. These observations have been confirmed by radioimmunoassay (25) and immunofluorescence (23, 26, 28). To test whether terminal transferase is synthesized by thymus and bone marrow, cell suspensions from these organs were incubated *in vitro* with [^{35}S]methionine and extracts were immunoprecipitated as previously described. Both thymus and bone marrow showed a M_r = 60,000 protein precipitable with immune, but not with normal, serum (Fig. 7). The band in bone marrow had approximately one-tenth the radioactivity as that from thymus, showing that the steady state levels of enzyme (6) correlate well with the synthetic rate. Also in agreement with measurement of steady state concentration was the fact that no synthesis of TdT-60 was evident in spleen cell suspension (data not shown).

It should be emphasized that the data in Fig. 7 was obtained by lysing cells in the presence of the protease inhibitor phenylmethylsulfonyl fluoride. If it was omitted, no labeled TdT-60 was recovered from bone marrow. This result is in agreement with our earlier demonstration of proteolysis of terminal transferase in human bone marrow samples (7).

Effect of Cortisone on TdT-60 Synthesis—Cortisone treatment of mice is known to kill most thymic lymphocytes over the course of 2 days, leaving a population of medullary thymocytes depleted for terminal transferase (6). The thymus is later repopulated with lymphocytes that contain terminal transferase (40). To investigate whether changes in terminal transferase synthesis in the thymus parallel the changes in detected enzyme activity, thymocytes from control or cortisone-treated mice were removed at various times and labeled with [^{35}S]methionine, and cell extracts were precipitated with anti-terminal transferase serum. At two days post-treatment, no M_r = 60,000 band was evident but, by about Day 4, synthesis of terminal transferase was again demonstrable and increases to normal levels over 7 to 10 days (Fig. 8). We have previously observed that terminal transferase activity in thymus is separated into two forms by phosphocellulose chromatography, peak I and peak II (6, 9). After cortisone treatment, peak II was reduced more than 50-fold on a per cell basis, but the level of peak I (originally representing 10% of the total terminal transferase activity) was reduced only 2-fold. If this remaining activity represented synthesis at the preceding rate, we would have expected to find 5% as much synthesis as in the original thymus. Synthesis was detectable in the remaining thymocytes at about 5% of the initial rate, suggesting that the peak I activity observed is in a cell type still synthesizing enzyme.

DISCUSSION

We have used a direct immunoprecipitation protocol (36) to study synthesis of murine terminal transferase in [^{35}S]-methionine-labeled cultured and primary cells. Because pure calf terminal transferase occurs as two polypeptides, we expected mouse terminal transferase to be a similar protein. When we found that a M_r = 60,000 polypeptide (TdT-60) was the only one precipitated by immune, but not by normal, serum, we designed a variety of tests to show that TdT-60 was truly terminal transferase and not an adventitious contaminant.

Presence of TdT-60 and enzymatically defined terminal transferase correlated in a variety of cell lines and tissues. TdT-60 and terminal transferase both eluted from phospho-

cellulose at about 0.27 M KCl and co-sedimented as M_r = 60,000 entities in sucrose gradients. Purified calf terminal transferase (the antigen used to raise the antiserum) blocked precipitation of TdT-60. (This circular experimental design would only be significant if no blocking was evident.) Finally, the antiserum specifically removed terminal transferase from a crude extract.

If TdT-60 is terminal transferase, then why the difference between calf terminal transferase and mouse terminal transferase? We are not yet sure of the answer but Johnson and Morgan (32) may have provided the crucial clue by showing that a purification scheme for calf terminal transferase which avoided some of the harsher aspects of the classical method (34) resulted in a M_r = 70,000 product. We have repeated their observation using another mild purification procedure.[5] Possibly the two-chain structure of the pure calf terminal transferase is an artifact of proteolytic degradation. It is also possible that calf terminal transferase is processed intracellularly to the two-chain form, but we could find no such processing of the murine enzyme. Murine TdT-60 had a half-life of less than 10 h in cultured thymoma cell lines (Fig. 6) but yielded no identifiable proteolytic fragments. In agreement with the recent report by Bollum and Brown (33), Silverstone and de Sostoa[6] have found that human terminal transferase is synthesized as a single polypeptide of about M_r = 60,000 in both human tumor tissue and in normal bone marrow and thymus.

The identification of terminal transferase by metabolic labeling and immunoprecipitation is a powerful tool with a sensitivity equal to enzymologic or immunofluorescent methods of detection. It gives information complementary to the other methods because it measures synthesis rate rather than steady state concentration. Like immunofluorescence, it measures enzyme physically but is less subjective than immunofluorescence.

The correlation we have found between terminal transferase enzyme and TdT-60 implies that none of the cells or tissues we have examined have detectable pools of inactive enzyme. Also, the detection of TdT-60 labeling in bone marrow at a 10-fold lower rate than in thymus implies that the bone marrow terminal transferase (which occurs at a 10-fold lower concentration than in thymus) can be synthesized in marrow cells and does not come from cells that migrate with their enzyme to the marrow as suggested by Pazmiño *et al.* (14).

One final use of this technique has been to confirm earlier reports (28) that lymphoid cells transformed by Abelson murine leukemia virus contain terminal transferase. These cells display some B-lymphocyte characteristics (35) and, thus, provide evidence that terminal transferase may occur in both T-lymphocyte precursors and B-lymphocyte precursors as previously suggested (28).

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[5] T. Martin, S. Mitra, and D. Baltimore, unpublished observations.
[6] A. E. Silverstone and A. de Sostoa, unpublished observations.